The three most downstream genes of the Hox-3 cluster are expressed in human extraembryonic tissues including trophoblast of androgenetic origin

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Summary

Human first trimester extraembryonic tissues of normal and androgenetic origin (molar pregnancies) were investigated for the expression of 6 homeobox genes from the chromosome 12-encoded Hox-3 cluster by non-autoradiographic in situ hybridization with biotinylated RNA probes. By comparative in situ hybridization involving the use of exon- or region-specific RNA probes, analysis included the cellular distribution of alternative Hox-3 transcripts in choriocamnion villous tissues. A bias in extraembryonic distribution was seen between transcripts of the three most upstream Hox-3 genes (Hox-3.7, -3.6, and -3.1) versus transcripts of the 3 most downstream genes (Hox-3.3, 3.4, and 3.5). Only genes from the latter group are transcribed in human extraembryonic tissues including extraembryonic tissues of androgenetic origin. Moreover, comparative in situ hybridization showed that distinct alternative transcripts of Hox-3.3, Hox 3.4 and Hox-3.5 are exclusively found in trophoblast cells while others are present in choriocamnion villous stromal cells as well. These data demonstrate the existence of tissue- and cell-specific use of transcriptional (alternative gene promoters) or post-transcriptional (alternative splicing) regulation of homeobox genes in extraembryonic tissues.

Key words: Hox-3, trophoblast, in situ hybridization, molar pregnancy, human embryo, gene expression.

Introduction

The embryonic forms of many vertebrates are much more closely similar than the resulting adult forms: embryonic development is characterized by evolutionary conservatism. The structures that are first to form serve as the framework on which later development must build; even a small mutation of the initial structures may upset many subsequent developmental processes that depend on them. Therefore, mutations affecting early development are seldom favored by natural selection, while at the same time the genetic mechanisms that establish the basic body plan are characterized by gene networks (the homeotic/segmentation network) found to be conserved amongst many different animals (Levine et al. 1984; McGinnis et al. 1984; Scott and Caroll, 1987).

However, the mammal takes a large detour by generating a complicated set of extraembryonic structures – notably the amniotic sac and the placenta – that enclose and protect the embryo and provide for the exchange of metabolites with the mother. It can be reasoned that development of trophoblast is characterized by the fact that parts of the fertilized mammalian egg are transformed into specialized (troph)ectodermal structures that have gained an entirely new position: outside the embryo (extraembryonic). By definition, because homeotic mutants are characterized by the transformation of parts of the body into structures appropriate for other positions, extraembryonic trophoblast determination and differentiation with subsequent placenta formation might have been accompanied in mammals by selective interference with genetic regulatory mechanisms operating in the homeotic/segmentation network. Positional imprinting in trophoblast is forced towards the generation of cells in a position outside the embryo where they are capable of coming into direct contact with genetically dissimilar maternal blood and tissues. Recently, we showed that lack of expression of HLA-B27 is retained in transgenic mouse trophoblast, indicating that conserved down-regulating genetic pressures working across species barriers underly control of classical major histocompatibility complex class I genes in extraembryonic development (Oudejans et al. 1989). In contrast, the expression and regulation of homeobox genes in trophoblast is largely unknown (Simeone et al. 1988), despite the fact that exploration of homeobox gene expression might
greatly facilitate investigation of homeobox gene regulation in mammals. Transcripts of murine homeobox genes have been identified so far mainly in extraembryonic tissue components generated from primitive ectoderm-derived extraembryonic mesoderm (allantois) (i.e. inner cell mass derivatives) (Sharpe et al. 1988; Gaunt, 1988). For most murine homeobox genes with exception of Hox-7 (Robert et al. 1989), no transcripts have been identified in trophoectodermal derivatives, either by Northern blotting (Colberg-Poley et al. 1985), by in situ hybridization (Sharpe et al. 1988; Gaunt, 1988) or by RNase protection assays (Jackson et al. 1985).

In this paper, investigation of early human extraembryonic tissues with a novel non-autoradiographic in situ hybridization procedure demonstrates that 3 (Hox-3.3, Hox-3.4 and Hox-3.5) from the 6 members investigated of the chromosome 12-encoded Hox-3 cluster are expressed in human trophoblast of normal origin as well of androgenetic (molar) origin. Moreover, by comparative hybridization with exon-specific probes, it is shown that certain alternative transcripts of these 3 homeobox genes are found exclusively in trophoblast, while others are present in choriocytional villous stromal cells as well.

**Materials and methods**

**Tissues**

**Extraembryonic tissues.** Human extraembryonic tissues of normal and androgenetic origin were obtained from first trimester curettings of normal and molar pregnancies, respectively (n=10; 5–9 weeks of menstrual age). In addition, normal extraembryonic tissues were obtained from tubal resections performed for reasons of tubal pregnancies (n=6; 6–8 weeks of menstrual age). The majority of complete hydatidiform moles arise through fertilization of an empty (anucleate) egg by a haploid sperm that subsequently duplicates. The complete chromosomal complement is therefore paternal in origin (androgenetic) and homozygous. The majority of invasive hydatidiform moles, characterized by the additional presence of villous structures in the uterus myometrium, is thought to arise by fertilization of an empty egg by two spermatozoa leading to a heterozygous (yet androgenetic) chromosome complement, the karyotype including 46XY. For both types of moles, mitochondrial DNA derived from the egg cytoplasm is maternal as controls. Histologically, no fetus is present while chorionic villi are characterized by marked trophoblast proliferation and villous hydroids (Szulman and Surti, 1978; Kajii and Ohama, 1977; Jacobs et al. 1980; Edwards et al. 1984). In man, extraembryonic tissues of gynogenetic origin do not exist and could therefore not be included. Tissue blocks were fixed in 4% buffered formaldehyde and processed as for routine histochemistry with embedding in low-melting-point paraffin (52–54°C) (Paraplast). The experimental data obtained by investigation of invasive hydatidiform moles will be presented elsewhere (Oudejans et al., in preparation).

**Hox-3 DNA clones**

1. **Locus-specific probes**

Plasmid DNA of pGEM-3 (Blue) vectors (Promega) containing 0.45 kb EcoRI–PstI cDNA inserts of the human Hox-3.1 gene (clone cpE45/36), 0.5 kb EcoRI–HindIII cDNA inserts of Hox-3.2 (c8) gene (clone c824) (formerly called Hox-6.1) (Sharpe et al. 1988; Kongsvanw et al. 1988), 0.28 kb EcoRI–Smal cDNA inserts of Hox-3.4 (cp11) (clones cp11/12 and cp11.4), and 0.5 kb EcoRI–HindIII cDNA fragments of Hox-3.5 (cp19) (clone cp8.10/33) (Fig. 1) were isolated by alkaline hydrolysis from E. coli JM109 cells. Inserts had been obtained from cDNA libraries of SV40-transformed fibroblasts and from human full-term placenta in lambda gt11 (Simeone et al. 1987, 1988; Cannizzaro et al. 1987). Plasmid DNA containing 0.27 kb gDNA inserts of the human Hox-3.6 gene (clone 20.3/3.30), 0.35 kb gDNA inserts of Hox-3.7 (clone CM 30.7/2), isolated from a human genomic library, were obtained identically.

2. **Alternative transcript-specific probes** (Figs 2 and 3)

Plasmid containing 0.24 kb EcoRI–XhoI cDNA (clone 11.7/47) or 0.490 EcoRI–EcoRI (clone 11.7/11) inserts of the 5-common exon shared by 4 transcripts (Fig. 2) of the Hox-3 cluster, 0.22 kb Alu–PstI cDNA (clone 21.3/520) or 0.170 kb HaeIII–HaeIII (clone 21.3/102) inserts of the cp11T transcript (Hox-3.4), 0.24 kb cDNA inserts of the cp8 transcript (Hox-3.5) (clone cp8.300/4), and 0.150 kb cDNA inserts of the 5-alternative exon used by the c8-long transcript (Hox-3.3) (clone T218/3) were obtained in similar ways.

**Biotinylated RNA probes**

Biotinylated RNA transcripts (sense and anti-sense) were generated from coding and non-coding strands according to Melton et al. (1984) by using biotin-11-UTP or allyl-amine UTP (Enzo Biochemicals, NY) and SP6 or T7 RNA polymerase (Promega Biotec). Reaction mixtures contained 40 mM Tris, pH7.6, 5 mM magnesium chloride, 10 mM DTT, 2 mM spermidine, 200 units of human placental ribonuclease inhibitor (RNAsin) (Promega), 0.4 mM each of ATP, GTP, and CTP, 1 mM of biotin-11-UTP (Enzo) or 0.4 mM allyl-amine UTP (Enzo), 1 μg of linearized DNA template and either 5 units of SP6 or 20 units of T7 RNA polymerase (Promega) for allyl-amine-UTP-labeled RNA probes and 15 units of SP6 or 25 units of T7 RNA polymerase for biotin-11-UTP-labeled RNA probes. For transcription reactions driven by T7 RNA polymerase, magnesium chloride concentration was adjusted to 8 mM and sodium chloride was added to 25 mM. After incubation for one hour at 37°C, an equal amount of enzyme was added followed by additional incubation for one hour. Templates and unincorporated nucleotides were removed by affinity-purification on Qiagen tip-5 tubes (Diagen GmBH) according to the manufacturers instructions except that no urea was used. After precipitation with isopropanol, probes labeled with biotin-11-UTP were dissolved in 20 μl of 10 mM Tris, pH 8.0, 1 mM EDTA containing 80 units RNasin mL⁻¹. Probes labeled with allyl-amine UTP were dissolved in 50 μl of 0.1 M sodium borate buffer, pH 8.5, and allowed to react for 2 h at 25°C after addition of 10 μl of CAB-NHS (Gibco BRL) (10 mg mL⁻¹ in dimethylformamide). After precipitation, probes were dissolved as above. Completion of linearization, and size and integrity of RNA transcripts were checked by gel electrophoresis and dot spot analysis.

**Tissue hybridization**

In situ hybridization on tissue sections was done non-autoradiographically yet sensitively and specifically by using biotinylated RNA probes, detection by streptavidin-labeled gold/silver enhancement and visualization by confocal laser scan microscopy with reflex contrast. The pictures generated by our technique are similar to dark-field pictures of autoradiographic tissue hybridization. The non-autoradiographic
procedure described by us, however, is superior to autoradiographic procedures with respect to spatial resolution and speed (total procedure one day). The validity of our non-autoradiographic in situ hybridization procedure has been demonstrated previously on (extra)embryonic tissue sections of transgenic mice harbouring the human HLA-B27 gene (Oudejans et al. 1989). For this, formalin-fixed, paraffin-embedded tissue sections (3μm thickness) on freshly coated slides were dried overnight at 37°C. To deparaffinized extensively by overnight incubation in xylol at 50°C, followed by progressive rehydration. After pretreatment in Lugol’s iodine and sodium thiosulfate, sections were treated with proteinase K (Boehringer Mannheim) (5–10μg ml⁻¹ in 50 mM Tris, pH 7.6, 5mM EDTA) for 15 min at 37°C, washed for 3 min in phosphate-buffered saline, pH 7.4 (PBS), and postfixed with 4% (w/v) paraformaldehyde in PBS for 10 min at room temperature. Paraformaldehyde fixative should be prepared freshly. After washing twice in PBS for 5 min each, hybridization mixture (100μl cm⁻²) containing previously determined optimal concentrations of biotinylated probes, 50% (v/v) deionized formamide, 2×SSC (0.3 M sodium chloride, 0.03 M sodium citrate, pH 7.5), 10% (w/v) dextran sulphate, and Bacillus RNA (50 μg ml⁻¹) was applied to the sections followed by heating for 8 min at 65–70°C. Hybridization was allowed to occur for 2 h at 50°C. No coverslips were used during hybridization. Dehydration was not performed at any experimental stage, because this was found to improve retention of RNA. Following hybridization, sections were washed for 60 min in 0.1×SSC at 68°C. After equilibration for 5 min at room temperature in 100 mM Tris, pH 8.0, 1 mM EDTA, and 0.5 M sodium chloride (RNase A buffer), sections were treated with RNase A (Boehringer Mannheim) (20 μg ml⁻¹ in RNase A buffer) for 30 min at 37°C. After this, sections were washed once again in 0.1×SSC for 30 min at 68°C. After equilibration for 5 min at room temperature in PBS, RNA–RNA hybrids were detected immunocytochemically by successive incubations with a 1:250 dilution of rabbit anti-biotin immunoglobulins (Enzo Biochemicals, NY) for 30 min, a 1:250 dilution of biotinylated goat anti-rabbit immunoglobulins (Vector Laboratories, Burlingame, CA) for 30 min, and a 1:100 dilution of colloidal gold (5 nm)-labeled streptavidin (Jansen Life Sciences Products, Belgium) for 60 min. To enhance the stability, the latter complexes were diluted in 50 mM Tris, pH 8.2 containing 2.5% (w/v) sodium chloride and 1% (w/v) bovine serum albumin (BSA). All incubations were followed by washes in two changes of PBS containing 0.05% (v/v) Tween-20 for a total time of 10 min. To ensure retention of gold molecules during subsequent silver enhancement, sections were postfixed in phosphate-buffered paraformaldehyde (4% w/v) for 10 min at room temperature and washed in PBS for 5 min. After washing in 3 changes of excess ultrapure water, silver enhancement was performed by the silver lactate/hydroquinon method according to the manufacturers instructions (Jansen Life Sciences, Belgium) using the IntenSe M system except that enhancement was done twice for 9 min each at 15°C. For controls, sense probes were included. No signal was seen when these probes were applied, except for a consistent signal over liver tissue due to the presence of endogenous biotin molecules. Liver was therefore excluded from interpretation. Finally, sections were counterstained with hematoxylin, dehydrated and embedded in Depex.

Visualization
Microscopic evaluation was done by conventional light microscopy and confocal laser scan microscopy with reflex contrast. The laser scan microscope used by us is a type II laser scan microscope (Zeiss, Oberkochen, Germany) equipped with a synchronously scanned pinhole diaphragm in the detector beam path, a servo-controlled galvanometer scanner and a dual laser system (Ar, 488 nm; HeNe, 633 nm). Images were visualized with the blue laser and an anti-flex lens (x63) (Zeiss). Only a x63 aperture anti-flex lens is commercially available. The advantages of confocal laser scan microscopy over conventional light microscopy have been described elsewhere (Baak et al. 1987). Resolution in lateral direction is about 0.2 μm and depth of field is 0.6–0.8 μm using high numerical aperture lenses.

Results
Human extraembryonic tissues of normal and androgenetic origin from first trimester (5–9 weeks of menstrual age) specimens were investigated for the transcription of 6 homeobox genes from the chromosome 12-encoded Hox-3 cluster (Fig. 1) by a novel non-autoradiographic in situ hybridization procedure (Oudejans et al. 1989). In situ hybridization for individual genes of the Hox-3 cluster was followed by comparative hybridization with exon-specific probes for the in situ identification of alternative transcripts from 4 Hox-3 genes (Figs 2 and 3).

Chorionic villous tissue
The data obtained after hybridization of chorionic villous tissues are shown in Figs 4 and 5. Although similar results were obtained in placental tissues of normal and adrogenetic (molar) origin, a clear bias is seen between expression of the three most upstream (Hox-3.7, Hox-3.6, and Hox-3.4) of the Hox-3 cluster.

<table>
<thead>
<tr>
<th>CLONES</th>
<th>orientation</th>
<th>size (μm)</th>
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<tr>
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<td>3.5</td>
</tr>
<tr>
<td>cp11/12</td>
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<td>3.5</td>
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<td>T 240</td>
<td>240</td>
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<tr>
<td>T 218/2</td>
<td>S — — T</td>
<td>150</td>
<td>3.3</td>
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Fig. 1. Schematic presentation of Hox-3 DNA clones together with the chromosomal organization of the Hox-3 cluster. Orientation of inserts relative to SP6 and T7 promoters in pGEM-3 (blue) vectors and size of inserts is shown.
Out of the 6 Hox-3 genes tested, only genes from the latter group are expressed in human placental tissues (Figs 4/5 and Table 1). In addition, although several alternative transcripts are found exclusively in chorionic villous trophoblast (Fig. 5), others are present in chorionic villous stromal cells as well (Fig. 6).

Discussion

The present study describes the cellular distribution pattern of transcripts from the Hox-3 homebox cluster in human extraembryonic tissues (Figs 1–6). Extraembryonic tissues included tissues of androgenetic origin (molar pregnancies). In both normal and androgenetic tissues, three out of the six Hox-3 members investigated were found to be transcribed in early trophoblast of normal as well as of androgenetic (mo-
and Kirk, 1985). This functional division is not as absolute as originally suggested. The most significant effect of genomic imprinting is that neither the male nor the female genome is by itself totipotential; maternal chromosomes are evidently needed for development while embryonic cells are totipotential or pluripotential, whereas paternal chromosomes appear to be required for proliferation of progenitor cells of differentiated tissues (Surani, 1989). Hence, parental chromosomes maintain a balance between growth and differentiation of embryonic and extraembryonic cells. Consequently, functional differences in embryonic and extraembryonic tissues between paternal and maternal chromosomes caused by genomic imprinting are linked to the stage of differentiation. In mouse, genetic experiments involving intercrosses between animals with specific duplications or deficiencies of chromosomal regions allowed examination of the effects of maternal or paternal duplications/deficiencies of specific chromosomal regions in genetically balanced mice. In this way, chromosomes 7, 11 and 17 were found to show preferential maternal activity. Preferential paternal activity was observed for chromosomes 2, 6, 7, 8 and 11. Others have been excluded (chromosomes 1, 4, 5, 9, 13, 14, 15), while for several autosomal chromosomes (3, 12, 16, 19) the data are uncertain (Searle and Beechey, 1985; Surani, 1986). Interestingly, mouse chromosomes 6, 11 and 2 harbour the Hox-1, Hox-2 and Hox-4 clusters, respectively. This raises the intriguing possibility that homeobox genes might be subject to genomic imprinting. Moreover, our finding that homeobox genes are expressed in extraembryonic tissues of androgenetic origin suggests this phenomenon might be operational in man as well. However, based on the paternal/maternal deficiency experiments, the chromosome that harbors the murine Hox-3 loci (chromosome 15) is thought not to be subject to differential activity. At first sight, this fact seems to
provide stronger evidence against our hypothesis than our own data (expression of Hox-3 homeobox genes in trophoblast of androgenetic origin) provide in favour of it. However, genetic deficiency experiments as done in mouse might give an underestimation of the frequency of loci subject to genomic imprinting. This especially holds true for homeobox genes which are known to consist of large transcription units (Simeone et al. 1988) likely to be dysregulated by the type of experiments described above. Instead, in our opinion direct proof should be obtained by examination of developmental stage-related tissue-specific variations in genomic methylation patterns and/or appearances of parental transcripts in transgenic mice heterozygous for homeobox alleles. So far, no polymorphic differences have been noticed between homeobox alleles that would allow experiments of the same discriminative nature in man as well, such as should be possible in partial molar pregnancies. Nevertheless, our present data showing expression of particular homeobox genes in human extraembryonic tissues of normal and androgenetic origin will facilitate future research in this direction.

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Hox-3 gene transcription in human trophoblast


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